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Phosphorylation of the Protein Encoded by the First Open Reading Frame of the MDG4 Transposable Element (*gypsy*) by Homologous and Heterologous Casein Kinases Type 2

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Abstract—Homogeneous casein kinase type 2 (CK2) was obtained from oocytes of *Rana temporaria* and cells of *Drosophila melanogaster* by chromatography on heparin-Sepharose, phosphocellulose, and Mono Q columns using a Pharmacia FPLC system. The procedure was first successfully used for the purification of CK2 from the *Drosophila melanogaster* cell culture. It has been shown that the protein encoded by the first open reading frame (ORF) of the *gypsy* transposable element (MDG4) is an effective protein substrate both for homologous and heterologous CK2 from the oocytes of *Rana temporaria in vitro*. Both enzymes catalyze the incorporation of two moles of phosphate per mole of protein. The K_m and V_{max} values for the reaction catalyzed by CK2 from the *Drosophila* cell culture were 32.5 ± 2.1 nM and 70.97 ± 1.89 nmol/min per μg, respectively, and for CK2 from oocytes, these values were 37.6 ± 2.8 nM and 66.02 ± 2.15 nmol/min per μg, respectively.

Key words: casein kinase type 2, retrotransposon, *gypsy*, GAG, phosphorylation substrates

Phosphorylation of proteins plays an important role in regulation of cellular functions including the regulation of genome expression in eukaryotes at all levels, from DNA repair and replication to translation of mRNA. A considerable part of the total phosphoprotein transferase activity in cell extracts is associated with the so-called casein kinases. A casein kinase type 2 has been found in all cell species in different groups of organisms; it exhibits wide substrate specificity *in vitro*. The acidic character of the protein and a minimal required Ser(Thr)-X-X-Glu(Asp) region are the sufficient attributes of the CK2 substrates [1, 2]. It is apparent that not all proteins phosphorylated by CK2 *in vitro* are its natural substrates; therefore, the identification of true cellular protein substrates for the enzyme is of significance.

The primary structure analysis of the enzyme subunits isolated from different sources, from yeasts to humans, has explained some properties of CK2, such as the ability of the enzyme to interact with nucleic acids [3, 4]. Control of nucleic acid (NA) functions is probably the key intracellular role of CK2. The enzymes and protein factors involved in expression and control of the eukaryotic genome comprise the major group of presumed CK2 substrates *in vivo* [2, 5]. Interactions with NA are the main characteristic property of these protein substrates, and their phosphorylation, often mediated by CK2, acts as a modulating mechanism on their NA-binding activity [6-10].

A GAG-like protein encoded by the first open reading frame (ORF1) located from the beginning of the coding region of the *gypsy* MDG4 transposable element is probably a natural CK2 substrate. It has been suggested that this protein (or its processing products) plays a crucial role in the formation of the *gypsy* virus-like particles (VLP), due to its ability to interact with *gypsy* RNA [11-17]. This work is focused on the analysis of phosphorylation of the polyprotein encoded by the *gypsy* ORF1, presumable a natural CK2 substrate, by homologous and heterologous CK2. Preliminary computer analysis showed that the GAG-like protein encoded by the *gypsy* ORF1 contains several potential phosphorylation sites for CK2; however, the phosphorylation mechanism has not been studied so far.

Abbreviations: CK2) casein kinase type 2; ORF) open reading frame; VLP) virus-like particles.

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MATERIALS AND METHODS

The following reagents were used: [γ-^{32}P]ATP with molar activity of 5,000 Ci/mmol was of domestic production (Obninsk); ATP was obtained from Sigma (USA); casein was purchased from Calbiochem (USA); heparin-Sepharose was from Pharmacia (Sweden); phosphocellulose P11 was obtained from Whatman (UK); and Mono Q for FPLC was obtained from Pharmacia (Sweden).

The standard buffer containing 10 mM triethanolamine, pH 7.8, 10 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 6 mM 2-mercaptoethanol, 10% glycerol, 100 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride was used for homogenization, and 0.1 mM buffer of the same composition was employed in column chromatography.

Drosophila melanogaster cell line S2 was grown on a standard medium containing 5% fetal calf serum (Sigma) under 5% CO_2. Cells (80 g) obtained from about 10 liters of the *Drosophila melanogaster* cell culture (10^7 cells/ml) were homogenized in 150 ml of the standard buffer containing 0.5 M NaCl, 1% Triton X-100, and 0.5 M NP-40.

Oocytes of *Rana temporaria* were washed in Ringer solution (6 g NaCl, 0.25 g KCl, 0.2 g $NaHCO_3$, and 0.3 g $CaCl_2$ in 2 liters of water) and disrupted with a Potter homogenizer (95 g in 150 ml of the standard buffer).

Further purification of CK2 to homogeneity from the *Drosophila* cells and frog oocytes was conducted at 4°C using the same columns, as described earlier [16].

Protein kinase activity was assayed in 100 μl of the standard buffer containing the protein fraction and 0.1 mM [γ-^{32}P]ATP (0.25 μCi per sample). If casein was used as a substrate, it was preliminarily dephosphorylated (heated in 0.1 M sodium-borate buffer, pH 9.5, for 10 min in a boiling water bath and adjusted to neutral pH). The precipitated protein was discarded by centrifugation, and the supernatant was thoroughly dialyzed against the standard buffer. Dephosphorylated casein was stored at −20°C and, when necessary, added to the reaction mixture (60 μg) and incubated for 1 h at 20°C. The reaction was stopped by the addition of cold TCA to a final concentration of 5%. After 30 min of incubation in the cold, the pellets were applied to GF/A glass filters (Whatman, UK), thoroughly washed with the same acid solution, and radioactivity was counted using the Cherenkov method using a SL-4000 scintillation counter (Intertechnique, France).

Cloning and expression in *E. coli* of the protein encoded by ORF1 of the transposable element *gypsy* MDG4 of *D. melanogaster* as well as the partial purification of the recombinant polypeptide were conducted as described earlier [17]. The standard buffer was supplemented with 8 M urea to prevent protein aggregation. In the presence of 8 M urea, the activity of CK2 was completely inhibited; therefore, if the ORF1-encoded protein was used as a substrate, the urea concentration in the preparation was decreased to 0.5 M by dialysis against decreasing urea concentrations (6, 4, 2, 1, and 0.5 M) in the standard buffer. The urea concentration in the samples containing different amounts of the protein under study in the standard incubation mixture varied from 0.5 to 17 mM. Such low urea concentrations had virtually no effect on the CK2 activity.

Phosphorylation of the recombinant protein using homologous (*Drosophila melanogaster* cell culture) or heterologous (*Rana temporaria* oocytes) CK2 was conducted in 100 μl of the standard protein kinase system for 1 h at 20°C. After incubation, 50 μg of BSA were immediately added to each sample followed by cold TCA at a final concentration of 5%. The pellets were transferred to the GF/A filters, and radioactivity was counted as described above. The amount of protein substrate in the sample was different, while the concentration of CK2 was constant and corresponded to the incorporation of 7 pmol ^{32}P/μg casein in 1 h at 20°C. Each point on plots represents an average value from five independent experiments.

Protein concentration in the samples was determined according to Schaffner and Weismann [18]. Ionic strength was measured on an OK-102/1 conductivity apparatus (Hungary). SDS-PAGE was performed in slabs of 10% polyacrylamide gels according to Laemmli [19]. A Pharmacia molecular weight kit (Sweden) containing phosphorylase *b* (94 kD), bovine serum albumin (67 kD), carbonic anhydrase (43 kD), soybean trypsin inhibitor (20.1 kD), and α-lactalbumin (14.4 kD) was employed.

The kinetic parameters of the equation for the initial velocity of the phosphorylation reaction were estimated by nonlinear regression. The data were analyzed with the Duggleby software package [20].

RESULTS

In our work, we used an effective three-stage procedure including affinity chromatography on heparin-Sepharose, chromatography on phosphocellulose P11, and FPLC on a Mono Q column. We were the first to use it for the purification of CK2 from *Drosophila melanogaster* cell culture; in the case of frog oocytes, it had already been used [21]. The original material was homogenized as described above to obtain a post-mitochondrial fraction; for the *Drosophila* cell culture, the concentration of NaCl was decreased to 0.1 M, and a ribosome-free extract was obtained, which was loaded on a heparin-Sepharose column (the column volume was 50 ml). The column was washed free of unbound proteins with 150 ml of the standard buffer, and proteins were eluted with an increasing linear NaCl gradient in the same buffer (the gradient volume was 320 ml). The CK2-containing fractions were combined, diluted with a low-ionic-strength buffer to 0.1 M NaCl, and applied to a phosphocellulose column (the column volume was 8 ml) prelimi-

narily equilibrated with the standard buffer. The column was washed free of unbound proteins in a similar manner, and the bound proteins were eluted with a linear NaCl gradient (0.1-1 M) in the standard buffer (the gradient volume was 60 ml). Protein concentration, ionic strength, and CK2 activity were measured in the fractions; those with the highest activity were pooled, diluted with low-ionic-strength buffer to reduce salt concentration to 0.1 M NaCl, and subjected to fast protein liquid chromatography (FPLC) on a Mono Q column. The CK2 from *Drosophila melanogaster* cells was eluted from heparin-Sepharose at NaCl concentration of 500 mM; from phosphocellulose, the enzyme was eluted as a broad peak with a NaCl gradient from 390 to 500 mM, and from the Mono Q column, the enzyme was eluted at 350 mM NaCl (Fig. 1).

Thus, using three consecutive chromatographic stages, we obtained a highly purified CK2 from *Drosophila melanogaster* cell culture; the enzyme was purified 185 times relative to the proteins bound to heparin-Sepharose. The specific activity of the preparation was 148 units/μg protein, and the recovery of the enzyme was 13.5% (Table 1). These results confirmed the efficiency of our purification procedure for *Drosophila melanogaster*. We also obtained a homogeneous CK2 preparation from the oocytes of *Rana temporaria*: the enzyme was purified 667 times relative to the heparin-Sepharose-bound proteins; the specific activity of the enzyme was 118 units/μg, and the yield was 12.9% (Table 2). Note that the degree of enzyme purification during heparin-Sepharose chromatography was taken for one unit of enzyme purification, and all subsequent values were estimated relative to this stage since it was impossible to accurately measure the CK2 activity in crude extracts [22].

The homogeneity of the CK2 and ORF1-encoded protein was monitored by SDS-PAGE. The purified enzyme from *Drosophila melanogaster* contained two polypeptides with molecular masses of 37 and 28 kD (Fig. 2a) corresponding to α- and β-subunits [23]. The enzyme from *Rana temporaria* oocytes consisted of three polypeptides with molecular masses of 43, 41, and 29 kD (Fig. 2b) corresponding to α-, α′-, and β-subunits of CK2, respectively [21]. The purified recombinant protein had a molecular mass of about 55 kD (Fig. 2b, lane *2*), this being in good agreement with the data obtained earlier [24].

The purified enzymes were assayed for phosphorylation of the recombinant protein encoded by ORF1 of the *gypsy* retrotransposon. The phosphorylation parameters for this protein by homologous and heterologous CK2 were rather similar. The maximum incorporation of ^{32}P into the protein substrate was 2 pmol per pmol protein in both instances, and the shapes of the saturation curves were identical (Fig. 3). To determine the kinetic parameters (K_m and V_{max}) of the phosphorylation reaction catalyzed by both CK2 preparations, the concentration of

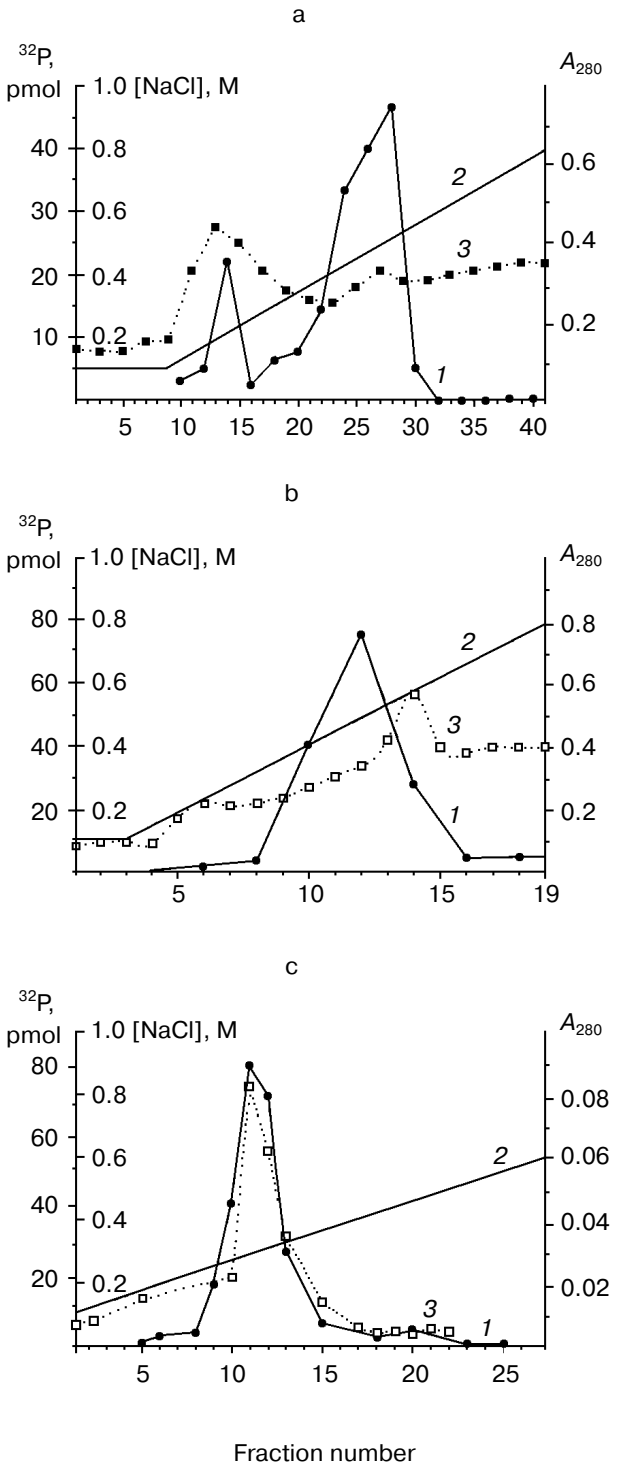

Fig. 1. Purification of the casein kinase type 2 from *Drosophila melanogaster* cell culture: a) chromatography on heparin-Sepharose; b) chromatography on phosphocellulose P11; c) FPLC on a Mono Q column (Pharmacia). To determine the protein kinase activity, each fraction (30 μl) was incubated with 60 μg of dephosphorylated casein and 0.1 mM [γ-^{32}P]ATP (0.25 μCi per sample) in the standard buffer for 1 h at 20°C. The total volume of the incubation mixture was 100 μl. *1*) Protein kinase activity; *2*) NaCl concentration in the samples; *3*) optical density at 280 nm.

Table 1. Purification of casein kinase type 2 from *Drosophila melanogaster* cell culture

Purification stage	Amount of protein, mg	Total activity × 10^{-3}, units	Specific activity × 10^{-3}, units/mg	Purification level	Yield, %
Heparin-Sepharose	10.24	8190	0.8	1	100
Phosphocellulose P11	0.172	3640	21.2	26.5	44
Mono Q	0.0075	1110	148	185	13.5

Note: Here and in Table 2, the amount of labeled phosphate (pmoles) incorporated during one hour of incubation with 0.1 mM [γ-^{32}P]ATP (0.25 μCi per sample) into 1 μg of dephosphorylated casein at 20°C in 100 μl of the standard buffer is taken as one unit of the CK2 activity.

Table 2. Purification of casein kinase type 2 from oocytes of *Rana temporaria*

Purification stage	Amount of protein, mg	Total activity × 10^{-3}, units	Specific activity × 10^{-3}, units/mg	Purification level	Yield, %
Heparin-Sepharose	15.4	2736	0.177	1	100
Phosphocellulose P11	0.168	1941	11.55	65.3	71
Mono Q	0.003	353	118	667	12.9

[γ-^{32}P]ATP was held constant (0.1 mM) while the protein concentration was varied from 20 to 300 nM. The relationship between the initial velocities of the phosphorylation reaction and protein concentration in a double reciprocal plot was nonlinear in both cases (Fig. 4), indicating that these reactions do not follow Michaelis–Menten kinetics. Computer analysis using the Hill equation produced Hill coefficients of 2.08 ± 0.25 for the *Drosophila* kinase and 2.18 ± 0.31 for the kinase from frog oocytes. The K_m and V_{max} values for the reaction catalyzed by CK2 from *Drosophila* calculated using the same algorithm were 32.5 ± 2.09 nM and 70.97 ± 1.89 nmol/min per μg, and for the CK2 from frog oocytes they were 37.6 ± 2.8 nM and 66.02 ± 2.15 nmol/min per μg, respectively.

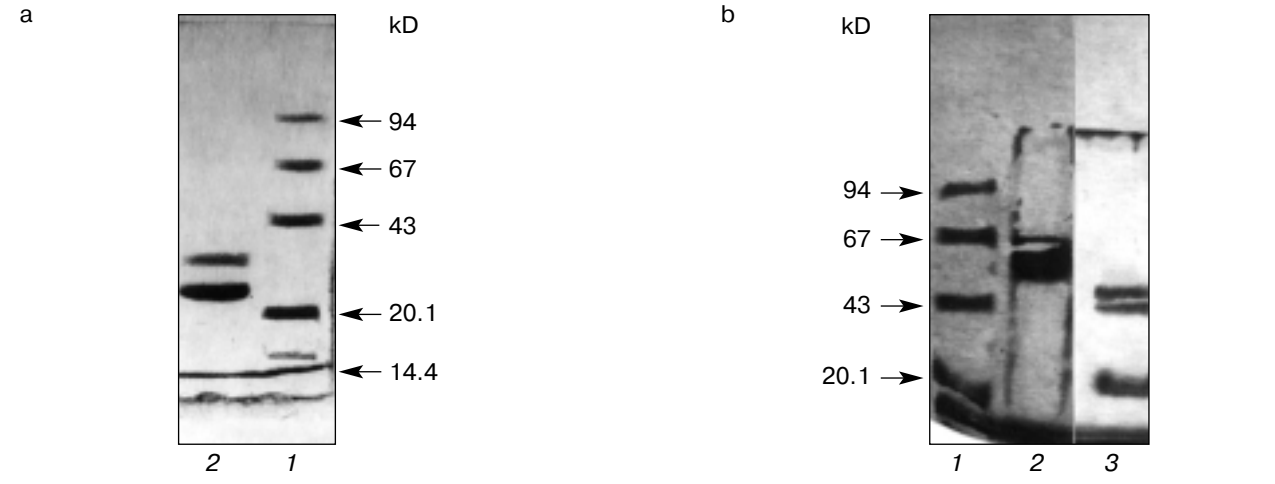

Fig. 2. SDS-PAGE: a) homogeneous preparation of casein kinase type 2 from cell culture of *Drosophila melanogaster*: *1*) molecular weight markers (shown in kilodaltons at the left); *2*) CK2 after chromatography on a Mono Q column; b) casein kinase type 2 from oocytes and a purified recombinant protein: *1*) low-molecular-weight markers (shown in kilodaltons at the left); *2*) ORF1-encoded recombinant protein; *3*) homogeneous CK2 preparation after chromatography on a Mono Q column. Gels were stained with colloidal Coomassie G-250.

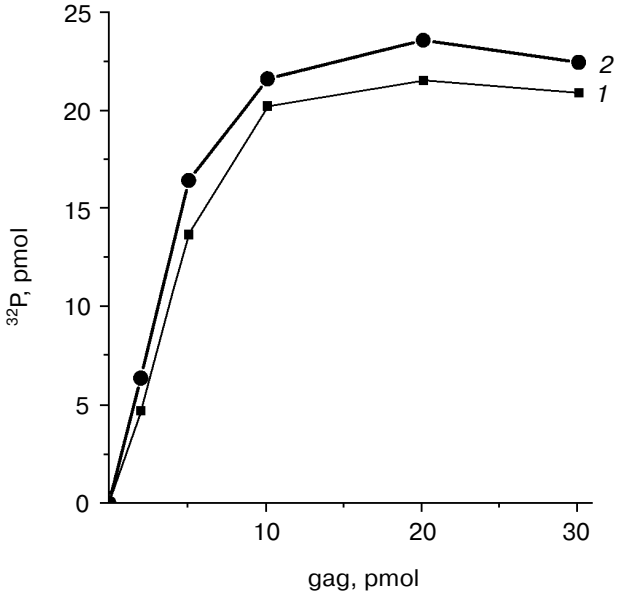

Fig. 3. Incorporation of radioactively labeled phosphate into the ORF1-encoded protein during the reaction catalyzed by: *1*) casein kinase type 2 from cell culture of *Drosophila melanogaster*; *2*) casein kinase type 2 from oocytes of *Rana temporaria*. The reaction mixture contained 0.1 mM [γ-^{32}P] ATP (0.25 μCi per sample), the casein kinase type 2 in the amount equivalent to the incorporation of 7 pmoles ^{32}P/μg casein (held constant in all samples), and the ORF1-encoded protein, whose amount was varied from 1 to 30 nmoles. The total volume of the reaction mixture was adjusted to 100 μl with the standard buffer. Incubation proceeded for 1 h at 20°C. Each point on the plot is the average of five independent experiments.

DISCUSSION

The CK2 from *D. melanogaster* is an $\alpha_2\beta_2$ heterotetramer, while CK2 from the oocytes of *R. temporaria* has $\alpha\alpha'\beta_2$ structure in which α and α' are the catalytic subunits, while β is a regulatory subunit [21, 23]. Despite the considerable difference in subunit composition, the chromatographic behavior of the two kinases was similar: the CK2 enzymes were virtually the same in their affinity for the sorbents used for enzyme purification. Using this unique procedure, homogeneous CK2 preparations from many taxonomically distant eukaryotic species such as the phytopathogenic fungus *Verticillium dahliae* Kleb and higher vertebrates have been isolated [16, 21, 25].

The GAG-like polyprotein encoded by the *gypsy* ORF1 was cloned and expressed in *E. coli* [24]. Preliminary computer analysis of the amino acid sequence of the recombinant protein deduced from cDNA indicated the presence of six potential phosphorylation sites (Ser-69, Thr-76, Thr-141, Thr-295, Ser-379, and Ser-401) for casein kinase 2. According to our data, both kinases phosphorylated the recombinant protein only at two sites, catalyzing the incorporation of two moles of phosphate per mole of protein. Thus, we first showed that the protein encoded by the *gypsy* ORF1 is an efficient protein substrate both for the homologous CK2 of *Drosophila melanogaster* and for the heterologous CK2 from the oocytes of the brown frog. The affinity of CK for this substrate was unexpectedly high, as compared to the majority of other potential intracellular protein CK2 substrates for which K_m values have been determined. The K_m values for these CK2 substrates varied from 0.39 to 100 μM (for example, 0.39 μM for phosphophorin; 0.5-2 μM for vitronectin; 0.5 μM for the Ca-binding protein S100B; 2.1 μM for osteopontin, and 100 μM for the T-substrate from wheat embryos) [26-29], while for the protein encoded by the *gypsy* ORF1, the K_m value was at least ten times smaller. This finding supports the suggestion that *in vivo* the GAG-like protein encoded by the *gypsy* ORF1 is predominantly phosphorylated by type 2 casein kinases.

In double reciprocal plots, the shape of the curves for the relationships between the initial velocities of the phosphorylation reaction of the GAG-like protein and its concentration in the reaction mixture is nonlinear (Fig. 4). At least two alternative suggestions could account for this phenomenon. First, this may be an artifact arising from the presence of different concentrations of urea during the enzyme activity assay. However, it cannot be excluded that

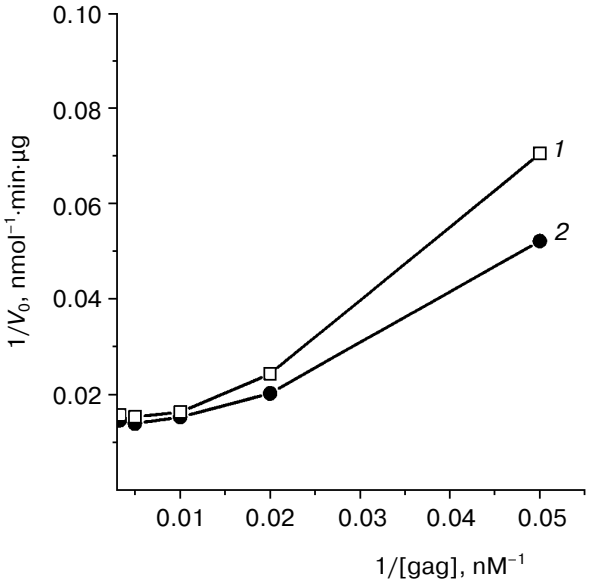

Fig. 4. Relationship between the initial rate of phosphorylation reactions and the concentration of the ORF1-encoded protein (double reciprocal plot): *1*) for the reaction catalyzed by CK2 from cell culture of *Drosophila melanogaster*; *2*) for the reaction catalyzed by CK2 from oocytes of *Rana temporaria*. The composition of the reaction mixture is given in the caption to Fig. 3. The incubation time was 15 min at 20°C. Each point on the plot represents the average value of five independent experiments.

the nonlinear character of the curves is due to positive cooperative interactions between CK2 and the unique protein substrate used. In all likelihood, binding of the substrate to one of the catalytic subunits of CK2 stimulates substrate binding to the second enzyme subunit. Positive cooperativity was reported for phosphorylation of the synthetic peptide RRREEETEEE in the presence of sphingosine by casein kinases 2 from rat brain [30]; however, for protein CK2 substrates, this phenomenon is shown for the first time. The mechanisms underlying this property of the enzyme deserves special investigation.

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